Suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling in infantile hemangioma

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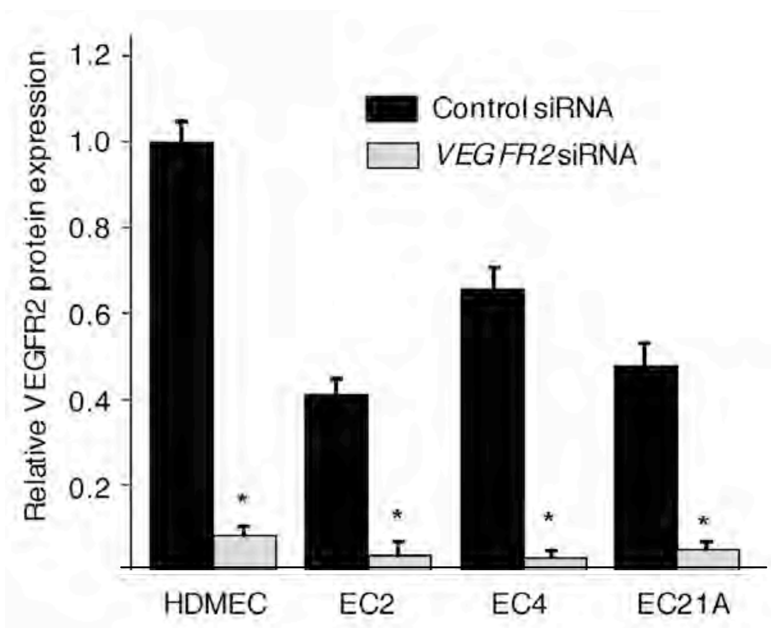
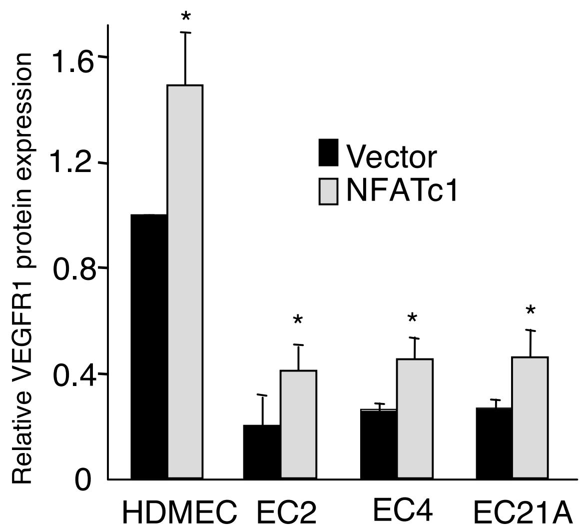
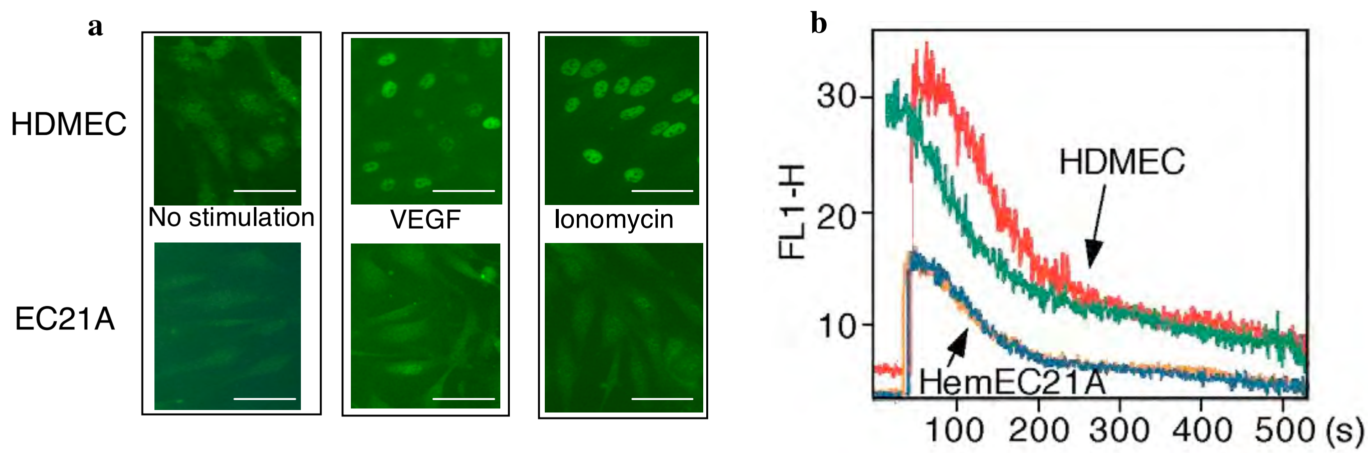


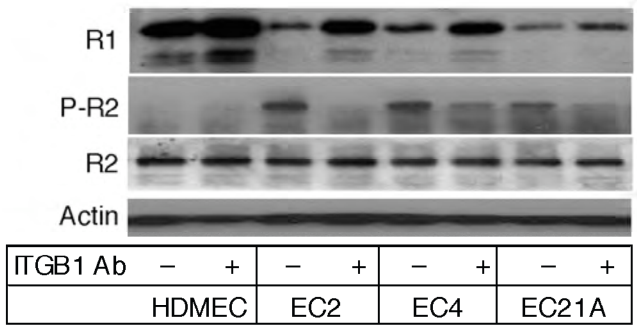
Supplementary Figure 1. VEGFR2 expression levels in endothelial cells
Relative levels of VEGFR2 protein expression in HDMEC (mean value set at 1.0) and hemEC lysates determined by quantitative multiplex ELISA. Treatment with *VEGFR2* siRNA reduces expression level to less than 10% in all cell types. *$P < 0.05$; error bars represent SD of +1 ($n = 3$).



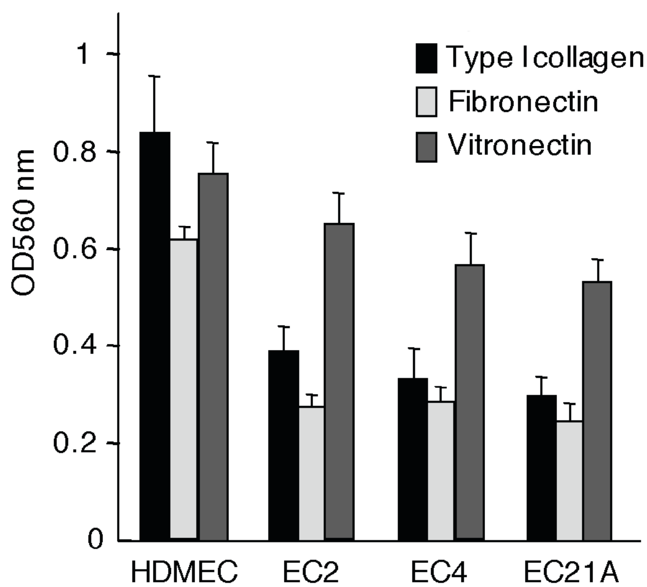
Supplementary Figure 2. NFAT activity increases VEGFR1 expression
VEGFR1 expression was increased by the overexpession of constitutively active NFATc1. VEGFR1 protein concentrations determined by ELISA in lysates of cells transfected with retroviral vector (black bars) or constitutively active NFATc1 (gray bars). *$P < 0.05$ as compared with the value in cells transfected with empty vector. Error bars represent SD of +1 ($n = 3$).



Supplementary Figure 3. NFAT and Ca^{++} in endothelial cells
(**a**) Staining for NFATc2 in HDMEC and hemEC21A cultures. The cells were treated with 1 mM EDTA for 15 min to chelate Ca^{++} and then left unstimulated or stimulated for 20 min by 50 ng ml^{-1} VEGF or 1 μM ionomycin in the presence of 10 mM $CaCl_2$. Similar results were obtained with hemEC from all nine patients. White scale bars are 50 μm.
(**b**) Increased fluorescence of the cytoplasmic Ca^{++} probe Fluo-4 following stimulation of HDMEC (green and red) and hemEC21A (orange and blue) with ionomycin (1 mM). For the green response curve, the recording did not capture prestimulation Ca^{++}-induced fluorescence.



Supplementary Figure 4. Effects of stimulatory antibody to β1 integrin
Treatment of hemEC with β1 integrin stimulating antibody upregulates VEGFR1 expression and reduces phosphorylation of VEGFR2. Immunoblotting of HDMEC and hemEC cell lysates following no treatment (–) or treatment with (+) 10 mg ml^{-1} of β1 integrin stimulatory antibody (ITGB1 Ab) for 24 h.



Supplementary Figure 5. Adhesion of endothelial cells to extracellular matrix proteins
Adhesion of HDMEC and hemEC to plates coated with collagen I, fibronectin or vitronectin. For hemEC, the mean values were significantly lower ($P < 0.05$) than those for HDMEC in adhesion to collagen I and fibronectin. Error bars represent SD of +1 ($n = 4$).

Supplementary Table 1

Hemangioma endothelial cell isolates used for the experiments

Patient no.	**HemEC**	**Patient sex (F/M)**	**Patient age (months)**	**Location**	**Mutation**
1	EC1	F	8	Right eyelid	
2**	EC2	M	3	Unknown	VEGFR2
4	EC4	F	3.5	Right flank	TEM8
10	EC10	F	24	Forehead	
12	EC12	F	4	Left cheek	
17*	EC17B	F	10.5	Forehead	VEGFR2
21*	EC21A	F	9	Scalp	
24	EC24	F	5	Unknown	
26	EC26	F	5	Unknown	

*From patients 17 and 21, cells isolated from only one of two lesions were studied.
** Clinical details of patient 2 and characterization of EC2 are not described in Boye *et al.* Clonality and altered behavior of endothelial cells from hemangiomas. *J. Clin. Invest.* **107**, 745–752 (2001).

Supplementary Table 2

Protein phosphorylation levels compared to HDMEC

Probe	**HDMEC + VEGF**	**EC2**	**EC4**	**EC21A**
EGFR (Tyr869)	1.9 ± 1.3	1.2 ± 0.9	0.8 ± 0.4	1.6 ± 1.1
EGFR (Tyr915)	2.0 ± 1.6	1.4 ± 0.1	1.5 ± 0.4	0.7 ± 0.3
EGFR (Tyr1069)	1.1 ± 0.3	1.5 ± 0.2	0.8 ± 0.3	1.0 ± 0.6
EGFR (Tyr1125)	1.4 ± 0.2	1.5 ± 0.6	1.1 ± 0.7	1.9 ± 1.5
EGFR (Tyr1172)	0.2 ± 0.1	1.7 ± 0.9	1.3 ± 1.0	0.3 ± 0.1
FGFR1 (Tyr766)	1.3 ± 0.4	1.6 ± 0.8	1.3 ± 0.6	1.2 ± 0.8
FGFR2 (Tyr769)	1.6 ± 0.9	1.1 ± 0.5	1.5 ± 0.3	0.6 ± 0.3
FGFR3 (Tyr648)	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1

Values represent the mean ± SD ($n = 3$) fold increase compared to HDMEC in the absence of exogenous VEGF.

Supplementary Table 3

Polymorphisms and mutations identified

Genes screened	**Polymorphisms and mutations (in bold)**
ANGPT2	
bFGF	
β3 Integrin	L59P, P294P, V381V
CMG2	P357A
CUL2	M335I, T373T
KRIT1	
LYVE1	
PDGFB	
PGF	
PHD2	
RAS-A1	
TEM8	**A326T**
THBS4	
TGF-βR1	
TGF-βR2	
TIE-2	G285A, N560N, V600L, S654S, Q698Q, V795V, Y992Y, G1041G, E1171A, V1229V
TNFSF12	
VBP1	
VEGFD	L37L
VEGFR1	L779F, P1068P, Y1213Y
VEGFR1 promoter *	
VEGFR2	V297I, Q472H, **C482R**, L963V
VEGFR3	N149D, Q890H, P1066P, H1146R
VG5Q	I405I
VHL	

*Sequencing 1kb of the *VEGFR1* promoter with DNA from the nine hemEC cultures showed no sequence variations.

Supplementary Methods

Antibodies. Recombinant human VEGF165, recombinant human VEGFR-1 (Flt-1)-Fc chimeric protein and monoclonal anti-human VEGF antibody were obtained from R & D Systems. Antibodies specific for VEGFR1, VEGFR2, His-tag, HA-tag, NFATc1, NFATc2 and total β1 integrin were from Santa Cruz Biotechnology. Antibodies against phospho-p44/42 MAP kinase (Thr202/Tyr204), MAP kinase, phospho-Akt, Akt and phospho-VEGFR2 (Tyr1175) were from Cell Signaling Technology. Anti-actin antibodies were from Sigma. Rabbit antibody to TEM8 was from IMGENEX. Mouse antibodies to TEM8 and to VEGFR1 were from Abcam. Antibody to total β1 integrin was from Covance. Integrin β1-specific stimulatory antibody was from Millipore. Antibody to active β1 integrin (HUTS-21) was from Pharmingen.

Immunoblotting and immunoprecipitation. Cells were lysed in lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 1 mM calcium chloride, 1 mM manganese chloride, 10% glycerol, and 5 mM sodium orthovanadate). Immunoblotting and immunoprecipitation was performed as described by Jinnin, M. *et al.* α2(I) collagen gene regulation by protein kinase C signaling in human dermal fibroblasts. *Nucleic Acids Res.* **33**, 1337-1351 (2005). Detection was with Super Signal West Femto (PIERCE).

DNA affinity precipitation assay. Three oligonucleotides containing biotin on the 5'-nucleotide of the sense strand were used: 1) Positive control oligo 5'-TCGACAAAAGGCGGAAAGAAACAGTCATTTC-3', containing the proximal NFAT-AP1 site of the *COX-2* promoter (see Hernandez, G. L. *et al.* Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporine A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. *J. Exp. Med.* **193**, 607–620 (2001)); 2) WT *VEGFR1* oligo, 5'-GGGAGGCGGATGAGGGGTGGGGACCCCTTGACGT CACCAGAA-3', corresponding to nucleotides -110 to -68 of the *VEGFR1* promoter; 3) Mut *VEGFR1* oligo, 5'-GGGAGGCGGATGAGGGGTGGGTCACCCCTTGACGT CACCAGAA-3', with the mutated sequence underlined. These oligonucleotides were annealed to their complementary oligonucleotides.

Cell lysates were incubated with each oligonucleotide and streptavidin-agarose. The protein-DNA-streptavidin-agarose complex was loaded onto an SDS-polyacrylamide gel, as described by Yagi, K. *et al.* c-myc is a downstream target of the Smad pathway. *J. Biol. Chem.* **277**, 854-861 (2002). NFAT was detected on immunoblots with anti-NFATc1 or NFATc2 antibody (Santa Cruz).

Quantitative real-time Polymerase Chain Reaction (PCR). Total RNA was isolated and first-strand cDNA was synthesized using iScript (Bio-Rad) with random hexamer primers. Quantitative real-time PCR with a BioRad iCycler® used primers mixed with the PA011 master mix (Superarray, Frederick, MD). Primer sets for *VEGFR1*, *VEGFR2*, *MCP-1*, *DSCR1*, *COX-2*, *NFATc1*, *NFATc2* and *GAPDH* were purchased from Superarray. Specificity of reactions was determined by melting curve analysis. The relative fold changes of gene expression between each gene of interest were calculated by standard curve method. For each gene of interest, we analyzed 3–7 independent samples.

Mutation detection, sequencing and allele specific PCR. We designed cDNA/gDNA PCR and sequencing primers for sequencing the entire coding sequences of 24 genes (**Supplementary Table 3**).

We identified mutations in *VEGFR2* exon11 and *TEM8* exon13 of genomic DNA and cDNA in patients with hemangioma. We amplified *VEGFR2* exon11 using forward primer 5'−GATGTTTTATTTTTCTCTGA−3' and reverse primer 5'−AATCTTTTCCTTACTCTTGAC−3'; *TEM8* exon13 using forward primer 5'−ATGAGCCCCAGAATGAT−3' and reverse primer 5'−AAGCGGCCACCCCAGTTGC−3'. We also sequenced *VEGFR2* cDNA using forward primer 5'−GCGGGGCATGTACTGACGATTATG−3' and reverse primer 5'−GCAAACAGGTGTGGGCAACTCTC−3'; *TEM8* cDNA using forward primer 5'−CGGATTGCGGACAGTAAGGA−3' and reverse primer 5'−GCCGACGCATATTGTTGTTGAGAT−3'.

For amplification, annealing temperature was set at 50 °C for *VEGFR2* exon11, 63 °C for *TEM8* exon13, 61 °C for *VEGFR2* cDNA and 53 °C for *TEM8* cDNA. Cycle sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems).

Allele-specific primers (for the T or C allele) were designed for the *VEGFR2* exon11 mutation such that the last (3'-most) nucleotide matched the genomic DNA template sequence; the penultimate nucleotide corresponded to the SNP of interest, and matched only one of the two alleles (bold). An additional destabilizing mismatch was introduced at position –3 (relative to 3'end), such that it did not match the template for either allele (italics). These were combined with a common reverse primer as follows:

VEGFR2"T"-F: "GTGACAAACCCATACCC*A***T**G" *or*

VEGFR2"C"-F: "GTGACAAACCCATACCC*A***C**G", with

VEGFR2 common-R: "TGCCTCACATATTATTGTACCATCC"

The annealing temperature was set at 60 °C.

Allele-specific primers (for the G or A allele) for the *TEM8* exon 13 mutation were:

TEM8 "G"-F: "GTTCCATCCTGGCCATAGC"

TEM8 "A"-F: GTTCCATCCTGGCCATTAC"; with the common primer

TEM8 common-R: TGACTGCTGGTGACTCATGG"

The annealing temperature was set at 55 °C. Presence or absence of a band on agarose gel electrophoresis indicated the presence or absence of that allele in the template DNA.

Transfection and reporter assays. For transient transfection, cells were grown to 50–80% confluence in 6- or 24-well dishes in growth medium. Cells were transfected with promoter constructs, expression vectors, and empty vectors employing GenePorter2 (Genlantis). X-tremeGene siRNA transfection reagent (Roche Mannheim) was used for *VEGFR2* siRNA duplex (5′-AACCCGGAGTGACCAAGGATTGTACAC-3′) or control siRNA duplex (5′-TCCTTCCTCTCTTTCTCTCCCTTGTGA-3′). siRNA Transfection Reagent was used for *VEGFR1*, *VEGFR2*, *TEM8* or *NFATc2* siRNA (Santa Cruz). Control experiments showed transcript levels for each siRNA target gene to be reduced by > 80% (data not shown).

Activities of *VEGFR1* promoter-firefly luciferase and Renilla (to correct for variations in transfection efficiency) reporters were assayed using the dual luciferase system (Promega).

To generate recombinant retrovirus, the packaging cell line 293-GPG was transfected with pMXs constructs. Culture supernatant was collected several times after 5 days and stored at –80 °C. For infection, cells were plated in 10 cm dish. After 12 h, 4 ml of virus supernatant was added with 8 μg ml^{-1} polybrene.

Immunocytochemistry. For visualization of nuclear translocation of NFAT, cells were treated with 1mM EDTA for 15 min to chelate Ca^{++} and then stimulated by 50 ng ml^{-1} VEGF or 1 μM ionomycin in the presence of 10 mM $CaCl_2$. The cells were fixed for 20 min in 3% PFA and treated with 0.5% Nonidet P40, 1% Na azide in PBS and 10% fetal bovine serum. Staining was done with clone 67.1 NFATc2 antibody (provided by S. Feske) and FITC conjugated secondary antibody (Vector Laboratories). For detecting the expression of the activated (HUTS-21) and total β1 integrin (K-20), cells were fixed in 3.7% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 3 min, and blocked with 5% non-fat milk at room temperature. The cells were then treated with primary antibodies overnight at 4 °C and treated with secondary antibodies for 1 h at room temperature. Coverslips were attached to slides using Vectashield fluorescent mounting medium with DAPI (Vector Laboratories). Images were acquired using an 80i fluorescence microscope (Nikon).

Ca^{++} measurements. Adherent cells were loaded with 1 mM of the fluorescein-based calcium probe Fluo-4 (Molecular Probes, Invitrogen), for 30 min. The cells were trypsinized, washed and resuspended in Ca^{++} free Ringer's solution immediately before the measurement. A basal intracellular Ca^{++} reading was taken over 40–80 s. Ionomycin (Calbiochem) was added and Ca^{++} levels were immediately measured again over a period of 512 s, collecting data from approximately 1 x 10^6 events. Ca^{++} levels were recorded with Cell Quest Software on FACSCalibur and analyzed using Flow-Jo software (Tree Star Inc).

Cell adhesion assays. For assaying adhesion of HDMEC and hemEC to collagen I, fibronectin or vitronectin, single cell suspensions were prepared from cell cultures by treatment with PBS containing 5 mM EDTA for 20 min. Cells (1 x 10^5 cells/well) were transferred to CytoMatrix cell adhesion strips (Millipore) coated with human collagen type I, fibronectin or vitronectin and incubated for 120 min at 37 °C. After washing to remove unattached cells, the attached cells were stained with 0.2% crystal violet. The cell-bound stain was solubilized and the optical density (at 560 nm) was determined.